Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations

Jesse H. de León[1]*, Valerie Fournier[2,3], James R. Hagler[2] & Kent M. Daane[3]

[1]*United States Department of Agriculture, Agricultural Research Service, Subtropical Agricultural Research Center, Beneficial Insects Research Unit, 2413 E. Highway 83 Weslaco, TX 78596, USA,* [2]*United States Department of Agriculture, Agricultural Research Service, Western Cotton Research Laboratory, Phoenix, AZ 85040, USA and* [3]*Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720, USA*

Accepted: 5 December 2005

Key words: SCAR and mitochondrial markers, cytochrome oxidase, Pierce's disease, glassy-winged sharphooter, predation, gut content analysis, Homoptera, Cicadellidae

Abstract

To aid in identifying key predators of Proconiini sharpshooter species present in California, we developed and tested molecular diagnostic markers for the glassy-winged sharpshooter, *Homalodisca coagulata* (Say), and smoke-tree sharpshooter, *Homalodisca liturata* (Ball) (Homoptera: Cicadellidae). Two different types of markers were compared, those targeting single-copy sequence characterized amplified regions (SCAR) and mitochondrial markers targeting the multicopy cytochrome oxidase subunit genes I (COI) and II (COII). A total of six markers were developed, two SCAR and four mitochondrial COI or COII markers. Specificity assays demonstrated that SCAR marker HcF5/HcR7 was *H. coagulata* specific and HcF6/HcR9 was *H. coagulata*/*H. liturata* specific. COI (HcCOI-F/R) and COII (HcCOII-F4/R4) markers were *H. coagulata* specific, COII (G/S-COII-F/R) marker was *H. coagulata*/*H. liturata* specific, and lastly, COII marker (Hl-COII-F/R) was *H. liturata* specific. Sensitivity assays using genomic DNA showed the COI marker to be the most sensitive marker with a detection limit of 6 pg of DNA. This marker was 66-fold more sensitive than marker Hl-COII-F/R that showed a detection limit of 400 pg of DNA. In addition, the COI marker was 4.2-fold more sensitive than the COII marker. In predator gut assays, the COI and COII markers demonstrated significantly higher detection efficiency than the SCAR markers. Furthermore, the COI marker demonstrated slightly higher detection efficiency over the COII marker. Lastly, we describe the inclusion of an internal control (28S amplification) for predation studies performing predator gut analyses utilizing the polymerase chain reaction (PCR). This control was critical in order to monitor reactions for PCR failures, PCR inhibitors, and for the presence of DNA.

Introduction

The glassy-winged sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae: Proconiini), is a large xylem-feeding leafhopper that is a serious economic pest because it vectors a strain of *Xylella fastidiosa* (Wells) (Proteobacteria: Xanthomonadaceae), a bacterium that causes Pierce's disease in grapevines [*Vitis vinifera* L. and *Vitis labrusca* L. (Vitaceae)], as well as diseases in many other plants (Hopkins & Mollenhauer, 1973; Hopkins, 1989). *Homalodisca coagulata* are native to the southern United States, ranging from Florida to Texas and northern Mexico (Young, 1958; Turner & Pollard, 1959; Nielson, 1968; Brlansky et al., 1983; Redak et al., 2004). However, within the past 15 years, *H. coagulata* have established in California where they pose a serious threat to the wine and table grape industry (Sorensen & Gill, 1996). Recent genetic evidence by de León et al. (2004) demonstrated that *H. coagulata* that invaded California have their origins

*Correspondence: Jesse H. de León, USDA, ARS, Subtropical Agricultural Research Center, Beneficial Insects Research Unit, 2413 E. Highway 83, Weslaco, TX 78596, USA.
E-mail: jhleon@weslaco.ars.usda.gov

in Texas. Their data showed that the populations in the USA were genetically distinct, clustering into two main groups or clades, a 'south-eastern' and a 'south-western and western' clade. In addition, the data also suggested more than one 'founding event' in California. Prior to the arrival of *H. coagulata*, the most common sharpshooter vectors of Pierce's disease in California were native sharpshooters in the tribe Cicadellini: *Graphocephala atropunctata* (Signoret) (blue-green sharpshooter), *Carneocephala fulgida* (Nottingham) (red-headed sharpshooter), *Draeculacephala minerva* (Ball) (green sharpshooter), and *Homalodisca liturata* (Ball) (smoke-tree sharpshooter) (Varela et al., 2001; Redak et al., 2004). *Homalodisca coagulata* may not be a more 'efficient' vector of *X. fastidiosa* than the California sharpshooters (Hill & Purcell, 1995; Almeida & Purcell, 2003), but it is certainly a more important vector for other reasons (Redak et al., 2004).

Effective control of *H. coagulata* requires an areawide, multitactic pest management program. A major component of such an approach is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. A classical biological control program is currently in progress in California against *H. coagulata*, utilizing parasitoid species that attack *H. coagulata* egg masses (Triapitsyn et al., 1998; CDFA, 2003). However, little is known about the predaceous enemies that feed on eggs, nymphs, or adults of *H. coagulata* (Triapitsyn et al., 1998). Direct visual field observations of predation are difficult to obtain, and the field study of insect predation has often relied on indirect techniques for measurement and analysis. A sensitive approach to detect prey in predator gut contents is the use of monoclonal antibodies (MAb) in enzyme-linked immunoassays (ELISA) (Greenstone & Morgan, 1989; Hagler et al., 1991, 1993; Hagler & Naranjo, 1994; Symondson & Liddell, 1996; Schenk & Bacher, 2004). More recently, polymerase chain reaction (PCR)-based methods have been developed that allow for rapid detection of prey in predator gut contents (reviewed in Symondson, 2002; Harper et al., 2005). These methods include (1) sequence characterized amplified region-polymerase chain reaction assays (SCAR-PCR) (Paran & Michelmore, 1993), where random amplification of polymorphic DNA-PCR (RAPD-PCR) species-specific bands are excised from gels and sequenced, and primers are designed towards those DNA fragments (Agustí et al., 1999b, 2000); (2) targeting genes that are present in the cell in high copy number, such as, mitochondrial genes [cytochrome oxidase subunit genes I and II (COI and COII, respectively)] (Hoy, 1994; Chen et al., 2000; Agustí et al., 2003a, 2003b; Juen & Traugott, 2005), and nuclear Internal Transcribed Spacer regions (ITS1) (Hoogendoorn & Heimpel, 2001); and (3) a sensitive and efficient multiplex PCR procedure incorporating fluorescent markers (Harper et al., 2005).

The aim of this work was to develop species-specific molecular diagnostic markers that were specific towards the invasive *H. coagulata* and the closely related *H. liturata*. Ultimately, the markers developed here will be used to detect *H. coagulata* and/or *H. liturata* remains in the guts of field-collected predators (V Fournier, JR Hagler, KM Daane, JH de León, RL Groves, HS Costa & TJ Hennebery, unpubl.). Identifying the key predators of these sharpshooters will help towards establishing a conservation or augmentation biological control program and will be useful in identifying the impact of natural enemies in field studies. In addition, these markers will be useful in identifying any life stage of *H. coagulata* and/or *H. liturata*, even before they emerge from egg masses, thus saving time and money required to rear these insects to the adult stage for morphological identification.

Materials and methods

Insects

Four Proconiini sharpshooter species, viz., *H. coagulata*, *H. liturata*, *Homalodisca insolita* (Walker) (johnsongrass sharpshooter), and *Oncometopia orbona* (Fabricius) (blue sharpshooter), were collected and processed as described in de León & Jones (2004) and de León et al. (2004). Three Cicadellini sharpshooter species, viz., *G. atropunctata*, *Carn. fulgida*, and *D. minerva*, were obtained from laboratory colonies maintained in California, as described in Hill & Purcell (1995), and send to Texas in 95% ethanol or in the case of *D. minerva* placed in quarantine at the United States Department of Agriculture (USDA), Animal and Plant Health Inspection (APHIS), Mission Plant Protection Center, Moore Air Field, TX, USA. Insects were reared on bermuda grass [*Cynodon dactylon* L. (Poaceae)]. With the exception of *H. insolita* and *O. orbona*, the sharpshooters used in this study are present in California (Varela et al., 2001), and all of the sharpshooters are vectors of *X. fastidiosa* (Redak et al., 2004). Our goal was to develop markers selective towards *H. coagulata* and *H. liturata*, and the other species were used to test for cross-reactivity. Third instar green lacewing larvae *Chrysoperla carnea* (Stephen) (Neuroptera: Chrysopidae), adult earwig *Forficula auricularia* (L.) (Dermaptera: Forficulidae), and adult ground beetle *Calosoma* spp. (Coleoptera: Carabidae) were used as the model predators to test for the presence of *H. coagulata* eggs or adults in their guts. Lacewings were purchased from Rincon-Vitova Insectaries (Ventura, CA, USA), and earwigs and beetles were obtained from colonies reared at the University of California, Riverside, CA, USA. We used these predators to

confirm the effectiveness of the developed markers after predators had fed on *H. coagulata* or *H. liturata*.

The specificity of the developed molecular diagnostic markers was further tested with additional homopterans that generalists predators may also feed on, such as, aphids, whiteflies, scales, and variegated leafhoppers. The additional homopterans included were *Aphis gossypii* Glover (Homoptera: Aphididae), *Bemisia tabaci* Gennadius (Homoptera: Sternorrhyncha), *Coccus hesperidum* L. (Homoptera: Coccidae), and *Erythroneura variabilis* Beamer (Homoptera: Cicadellidae).

DNA isolation

High molecular weight genomic DNA was extracted from sharpshooters according to standard methods (Sambrook & Russell, 2001) and as previously described by de León & Jones (2004). Individual whole sharpshooters were homogenized on ice in 5-ml polystyrene round-bottom tubes in 2 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1% SDS] with one 20-s burst with a tissue homogenizer (Tissue Tearor, Biospec Products, Inc., Bartleville, OK, USA). The final DNA pellet was resuspended in 100 µl of TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 7.5)]. The integrity of the DNA was determined by gel electrophoresis on 1% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) in the presence of 0.2 µg ml^{-1} ethidium bromide. The concentration of the DNA was estimated with the DNA Mass Ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). The DNeasy Tissue kit from QIAGEN Inc. (Valencia, CA, USA) was used to extract DNA from whole predators following the instructions from the manufacturer on the protocol for isolation of total DNA from animal tissues. The insects were homogenized as described for sharpshooters. To accommodate for the sizes of different insects, the volumes of material used in each procedure were correspondingly scaled up or down.

For the lacewing larvae, a rapid crude DNA extraction procedure was tested (Black et al., 1992). The detergent Nonidet P-40, used in the original procedure, was replaced by IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO, USA). Individual lacewing larvae were homogenized on ice in 1.5-ml microfuge tubes in 60 µl of lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1% IGEPAL CA-630, 100 µg ml^{-1} Proteinase (K)] with one 30-s burst on ice (Pellet Pestle Motor, Bel-Art Products, Pequannock, NJ, USA). To avoid cross-contamination between samples, a single sterile plastic pestle was used for each insect. The samples were incubated at 95 °C for 5 min, followed by 1 min on ice. The samples were centrifuged for 10 min at 16,110 ***g*** at 4 °C. The supernatant was transferred to fresh microfuge tubes and stored at –20 °C.

To confirm for the presence of DNA in the samples, amplification reactions were performed with 1 µl of stock DNA and 28S primers at an annealing temperature of 65 °C (forward 5′-CCCTGTTGAGCTTGACTCTAGTCT-GGC-3′ and reverse 5′-AAGAGCCGACATCGAAGGATC-3′) (Werren et al., 1995) with 1.5 mM $MgCl_2$ and the amplification conditions described below. The 28S amplification reaction was also performed as an internal control on specimens where DNA was extracted by the other procedures. This was an important step because it allowed us to monitor the amplification reactions. If a diagnostic assay did not detect prey or produced positive banding and the 28S reaction did, then this would indicate that a negative amplification reaction was the result of the absence (or beyond the detection limits) of prey DNA rather than the presence of PCR inhibitors (or failures) (Vega et al., 1993; Pooler et al., 1997).

RAPD-PCR DNA fingerprinting

Primer pair-RAPD-PCR (pp-RAPD-PCR) of sharpshooters was performed as previously described (Yasukochi, 1998; de León & Jones, 2004) in a final volume of 20 µl with the following components: 1× PCR buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.0 mM $MgCl_2$, and 0.01% gelatin], 0.25 mM deoxynucleotide triphosphates, 0.25 pmol ml^{-1} decamer primers [OPA-03 (5′-AGTCAGCCAC-3′) and OPA-10 (5′-GTGATCGCAG-3′), Operon Technologies, Inc., Alameda, CA, USA], 0.5–1.0 ng of high molecular weight genomic DNA and 0.05 U ml^{-1} *Taq* DNA Polymerase (New England Biolabs, Beverly, MA, USA). The cycling parameters were as follows: 1 cycle at 94 °C for 2 min followed by 45 cycles at 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. Negative control reactions were performed in the absence of genomic DNA. Amplification products were loaded onto 2% agarose gels and submitted to electrophoresis in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA) in the presence of ethidium bromide. Photographs of the gels were taken with the Chemi Doc System and markers / bands were scored with the QUANTITY ONE software (Bio-Rad Laboratories, Hercules, CA, USA).

Sequence characterized amplified region marker development

An *H. coagulata*-specific RADP-PCR band (674 bp) was excised from the gel (QIAEX II Gel Extraction Kit, QIAGEN Inc., Valencia, CA, USA), subcloned with the TOPO Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA), plasmid minipreps were prepared by the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA, USA), and sequencing was performed by GENEWIZ INC (North Brunswick, NJ, USA). Sequences from two individuals were processed by the sequencing program SEQUENCHER version 4.1.4 (Gene Codes Co., Ann Arbor, MI, USA). SCAR markers or primers were designed

manually. All oligonucleotides were synthesized by Fisher-Sigma GenoSys (Houston, TX, USA).

Mitochondrial cytochrome oxidase subunit gene marker development

The general primers C1-J-1718 (forward: 5′-GGAG-GATTTGGAAATTGATTAGTTCC-3′) and C1-N-2191 (reverse: 5′-CCCGGTAAAATTAAAATATAAACTTC-3′) were utilized to amplify the COI partial gene from both *H. coagulata* and *H. liturata* (Simon et al., 1994; Agustí et al., 2003b). The 10× PCR buffer and assay conditions utilized are described previously (RAPD-PCR DNA fingerprinting) with the following modifications: 2 U of *Taq* DNA polymerase and 0.25 μM primers. The cycles parameters were as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Primers HcCOII-F (forward: 5′-CAGATTAGTGCAATGAATTT-AAGATTC-3′) and HcCOII-R (reverse: 5′-TTCTGAAC-ATTGACCAAAAAATAACCC-3′) were designed towards the *H. coagulata* partial COII gene from GenBank accession number AF456144 (Moran et al., 2003) and used to amplify both *H. coagulata* and *H. liturata* COII. The same 10× PCR buffer described previously was used with 1.5 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase. The cycling parameters were as follows: 94 °C for 3 min followed by 45 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s. DNA from two individuals of each species was sequenced in both the forward and reverse directions. Sequences were aligned with ClustalW (Thompson et al., 1994) from the DNASTAR program (Madison, WI, USA). *Homalodisca coagulata*-, *H. liturata*- and *H. coagulata*/*H. liturata*-specific primers were manually designed towards these sequences.

Sensitivity of molecular diagnostic markers

The detection limits of the diagnostic markers were tested by varying the amount of *H. coagulata* or *H. liturata* genomic DNA. Depending on the sharpshooter species and the diagnostic marker, the genomic DNA ranged from 0.00625 to 3.2 ng. Each point was performed in either triplicate or quadruplicate, averaged, and plotted vs. relative band density. Band densities were measured with the QUANTITY ONE software. Only the points that were within the linear range of the curve were utilized to determine the sensitivity limits of each diagnostic marker and sharpshooter pair. The graphs were constructed with GRAPHPAD PRISM 4.03 for Windows [GraphPad Software, San Diego, CA, USA; (www.graphpad.com)].

Detection efficiency of molecular diagnostic markers in predator feeding trials

To determine the utility and efficiency of the various molecular markers, we tested them with three generalist predators that fed on either *H. coagulata* eggs (lacewing and earwigs) or adults (ground beetles). Prior to the feeding trials, individual predators were placed in 4.0-cm diameter Petri dishes and starved (with ad libitum access to water) for 48 h. The lacewing and earwig individuals were fed six and three eggs, respectively, whereas the ground beetle fed on two adult *H. coagulata*. Immediately thereafter (0 h), the predators were frozen at −80 °C and later submitted to the QIAGEN preparation of DNA for each of the developed markers, as previously described. We also tested the detection efficiency for lacewing larvae at postfeeding intervals of 0, 4, 8, 16, and 24 h after feeding on *H. coagulata*. Prior to the feeding trials, individual lacewings were starved, as described previously. During the feeding period, lacewings were held under constant light at 25 °C, except for the 24 h interval in which the predators were held under a L16:D8 photoperiod. Additionally, *H. liturata*-specific markers were tested with lacewings feeding on *H. liturata* eggs and we also compared the efficiency of the two types of molecular markers, single-copy SCAR markers vs. multicopy mitochondrial targeted genes (COI, COII). After each feeding interval, the individuals were frozen at −80 °C and later submitted to the crude DNA extraction procedure. In all trials, negative controls included predators not fed on *H. coagulata*. Fisher's exact tests were performed using two-sided P values with GRAPHPAD INSTAT version 3.05 for Windows [GraphPad Software, San Diego, CA, USA; (www.graphpad.com)].

Results

Homalodisca coagulata, *Homalodisca liturata*, and *H. coagulata*/*H. liturata* molecular diagnostic markers

RAPD-PCR DNA fingerprinting was performed with various sharpshooters, and an *H. coagulata*-specific band (674 bp) was excised, sequenced, and SCAR markers were designed from it (data not shown). This RAPD-PCR band fragment was assigned GenBank accession number AY959333. Both *H. coagulata*/*H. liturata*- (HcF6/HcR9) and *H. coagulata*-specific (HcF5/HcR7) primer sets were designed from this sequence, and they produced amplification products of 166 and 302 bp sizes, respectively. Table 1 shows the optimized amplification reaction conditions for each diagnostic primer set and the name, the sequence of the primer, the expected amplification product size, the $MgCl_2$ concentration, the annealing temperature (Tm), and the number of cycles. The amplification reaction conditions are highly specific to each primer set in order to prevent cross-reactivity with any of the non-target species. If the specific reaction conditions are modified, those new conditions must be tested with all species of interest to test for cross-reactivity.

Sequencing of the COI gene fragments produced sizes of 525 and 524 bp for *H. coagulata* and *H. liturata*, respectively.

Table 1 Summary and optimized conditions of diagnostic primer sets showing primer name, sequence, DNA fragment size (bp), $MgCl_2$ concentration, annealing temperature (Tm), cycle number, and species specificity. F, forward; R, reverse; COII, mitochondrial cytochrome oxidase subunit gene II; COI, mitochondrial cytochrome oxidase subunit gene I; Hc, *Homalodisca coagulata*; Hl, *Homalodisca liturata*; G/S, *H. coagulata/H. liturata*

Primer name	5′-Sequence-3′	Frag. size	$MgCl_2$ (mM)	Tm (°C)	Cycle number	Designed towards
SCAR						
HcF5	AGGCAACGCAATGAACGGAAA	302	2.0	65	45	Hc
HcR7	AACGCAATGAACGGAAA					
HcF6	AAAGCCAAATGCTTCTTAATTGTTTT	166	2.0	59	45	Hc/Hl
HcR9	GAACTAGTAATGTTGCTTGCTAAATG					
Mitochondrial						
HcCOII-F4	CTTATAATTACTACAGTAGTTAGATAT	295	1.6	55	35	Hc
HcCOII-R4	GATTCTAAATTAATAGTTGGC					
G/S-COII-F	GCAGTATCCCCAATTATAGAAC	178	1.5	56	30	Hc/Hl
G/S-COII-R	CAGCTGGTATTTTAGTTCAAA					
HcCOI-F	GGGCCGTAAATTTTATTACC	197	1.4	60	31	Hc
HcCOI-R	ACCACCTGAGGGGTCAAAA					
Hl-COII-F	ATTATAATTACTACAGTAGTAAGATAC	295	1.6	56	33	Hl
Hl-COII-R	AATTCTAAATCAATAGTTGGT					

The GenBank accession numbers for these COI sequences are AY959334 for *H. coagulata* and AY959335 for *H. liturata*. Fragment sizes of 649 and 655 bp were produced for *H. coagulata* and *H. liturata* COII partial genes, respectively. GenBank accession numbers for the COII sequences are AY959336 and AY959337 for *H. coagulata* and *H. liturata*, respectively. About a 3.4% difference in nucleotides was seen between *H. coagulata* and *H. liturata* for each mitochondrial gene sequence, demonstrating the close genetic relationship between these two *Homalodisca* sharpshooters. An *H. coagulata*-specific primer set was developed towards the COI sequence, whereas two sets of primers were developed towards the COII sequence, an *H. coagulata*/*H. liturata*- and an *H. coagulata*-specific set. Lastly, a COII-specific primer set was developed towards *H. liturata*.

Species specificity of the molecular diagnostic markers

To test the specificity of the diagnostic markers, amplification assays were performed with stock genomic DNA from various sharpshooter species, several of them present in California, along with lacewing, earwig, and ground beetle predators. The results of the specificity assays for all six diagnostic markers that were designed towards the RAPD-PCR fragment and the COI and COII partial sequences are given in Figure 1. For the size of the expected amplification products and the specific reaction conditions of each marker refer to Table 1. As seen each diagnostic marker was highly specific towards its target(s) (Figure 1). All diagnostic markers amplified DNA fragments of the correct size and none crossed-reacted with other sharpshooter species or the predators of interest. The internal control, 28S amplification, is seen across all samples, indicating that PCR inhibitors or failures did not play a role in the reactions (Figure 1G).

Cross-reactivity assays with the additional homopterans

To confirm that the developed diagnostic markers did not cross-react with homopterans that the generalist predators are also likely to feed on, we preformed cross-reactivity assays with all six diagnostic markers using various homopterans. The homopterans tested in this study included cotton aphid, *A. gossypii*; citrus brown scale, *C. hesperidum*; whitefly, *B. tabaci*; and variegated leafhopper, *E. variabilis.* Figure 2 shows the results of a representative example using the *H. coagulata*-specific marker, HcF5/HcR7. The whole gel is shown to demonstrate that only the specific band of interest was amplified. Only one band of the correct size (302 bp) was seen with the *H. coagulata* sample. No cross-reactivity was seen with any of the additional tested homopterans. The same result was seen with all six developed molecular diagnostic markers (not shown).

Detection of *Homalodisca coagulata* DNA in predator guts

The results of the amplification assays of predators from the laboratory feeding trails showed that all diagnostic markers, *H. coagulata*/*H. liturata*-, *H. coagulata*-, and *H. liturata*-specific detected prey in predator gut contents (Figure 3A–E). As demonstrated, amplification was not seen in predators not fed on *H. coagulata*, whereas the 28S amplifications (Figure 3G) were positive. Sensitivity of

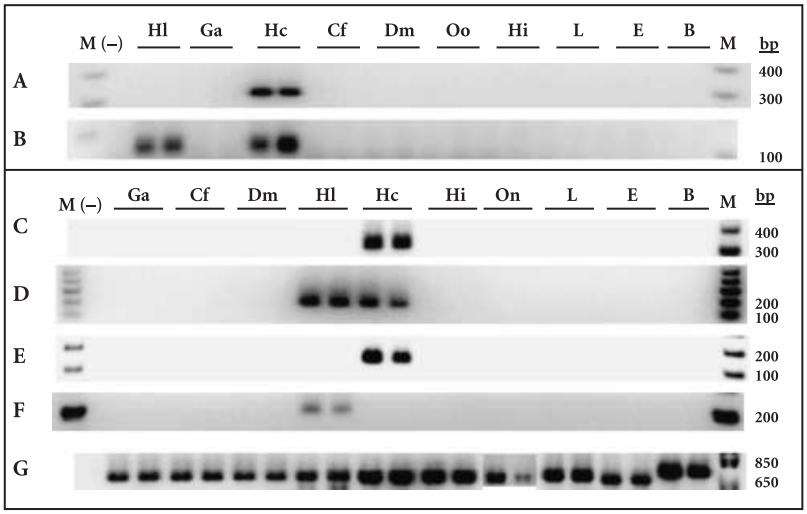

Figure 1 Specificity of molecular diagnostic markers. (A) and (B) RAPD-PCR DNA fingerprinting was performed with the following sharpshooters: *Homalodisca liturata* (Hl); *Graphocephala atropunctata* (Ga); *Homalodisca coagulata* (Hc); *Carneocephala fulgida* (Cf); *Draeculacephala minerva* (Dm); *Oncometopia orbona* (Oo); and *H. insolita* (Hi). The optimal amplification conditions for all reactions are listed in Table 1. (A) *Homalodisca coagulata*-specific marker, HcF5/HcR7 (302 bp) and (B) *H. coagulata*/*H. liturata*-specific marker, HcF6/HcR9 (166 bp). Predators included in the analysis were *Chrysoperla carnea* [green lacewing larvae (L); *Forficula auricularia* [earwig (E)]; and *Calosoma* spec. [ground beetle (B)]. (C) *Homalodisca coagulata*-COII-specific primers, HcCOII-F4/R4 (295 bp). (D) *Homalodisca coagulata*/*H. liturata*-COII-specific primers, G/S-COII-F/R (178 bp). (E) *Homalodisca coagulata*-COI-specific primers, HcCOI-F/R (197 bp). (F) *Homalodisca liturata*-COII-specific primers, Hl-COII-F/R (295 bp); (G) 28S internal control. (–), negative control (no template DNA). M: 1.0 Kb Plus DNA Ladder.

the diagnostic markers using stock genomic DNA is shown in Figure 4, which shows a representative example of the sensitivity assay: primer set HcF6/HcR9 and *H. coagulata* DNA. The sensitivity limits for our standard PCR procedure for the rest of the diagnostic markers are shown in Table 2 (numbers in brackets). The marker set targeting the COI gene, HcCOI-F/R exhibited the highest sensitivity (6.0 pg of DNA), whereas marker Hl-COII-F/R was the least sensitive (400 pg of DNA), a 66-fold difference. The difference in sensitivity between *H. coagulata* COI (HcCOI-F/R) and COII (HcCOII-F4/R4) was about 4.2-fold.

Efficiency of molecular diagnostic markers at detecting *Homalodisca coagulata* remains in the guts of predators

Between the two SCAR markers, marker HcF6/HcR9 was slightly more efficient than marker HcF5/HcR7; a significant difference was seen with lacewing at the 0 h time point (Table 2). Marker HcF6/HcR9 produced an amplification product size of 166 bp, whereas marker HcF5/HcR7 produced one of 302 bp, a difference of 136 bp. In lacewings at the 0 h retention interval, the percentage detection was 8.3 and 58.0% for marker HcF5/HcR7 and HcF6/HcR9, respectively. Because the detection efficiency was low for

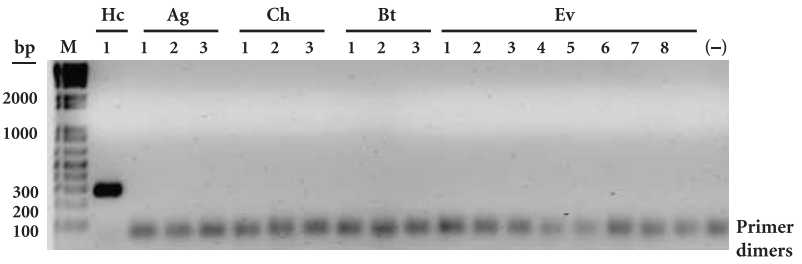

Figure 2 Sample cross-reactivity assay testing the additional homopterans that generalist predators may feed on. The whole gel is presented to show that the markers are detecting only the one band of interest. In this example the *Homalodisca coagulata*-specific molecular diagnostic marker HcF5/HcR5 (302 bp) was tested with the following homopterans: cotton aphid, *Aphis gossypii* (Ag); citrus brown scale, *Coccus hesperidum* (Ch); whitefly, *Bemisia tabaci* (Bt); and variegated leafhopper, *Erythroneura variabilis* (Ev). Hc, *H. coagulata*. (–), negative control (no template DNA). M: 1.0 Kb Plus DNA Ladder.

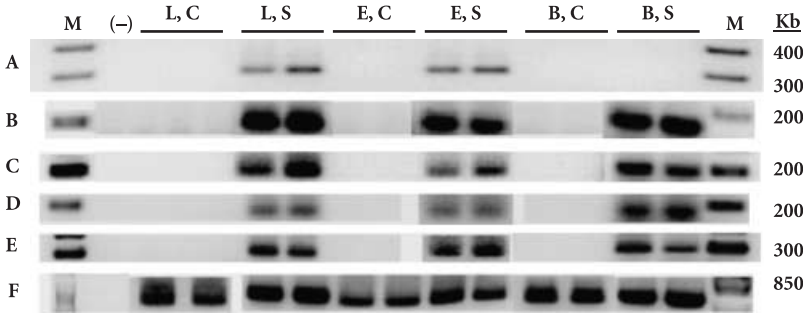

Figure 3 Detection of *Homalodisca coagulata* eggs or adults in predator gut contents by diagnostic amplification assays. (–), negative control (no DNA template); C, control (not fed on *H. coagulata*); S, sample (fed on *H. coagulata*). Lacewings and earwigs fed on *H. coagulata* eggs and ground beetles fed on *H. coagulata* adult(s). (A) HcF5/HcR7 (*H. coagulata*-specific; 302 bp); (B) HcF6/HcR9 (*H. coagulata*/*H. liturata*-specific; 166 bp); (C) HcCOII-F4/R4 (*H. coagulata*-COII-specific; 295 bp); (D) G/S-COII-F/R (*H. coagulata*/*H. liturata*-specific; 178 bp); (E) HcCOI-F/R (*H. coagulata*-COI-specific; 197 bp); (F) 28S internal control.

SCAR markers, we did not further test the rest of the time intervals. Detection of *H. coagulata* in earwig gut contents was equally low with both SCAR markers. *Homalodisca coagulata* could not be detected in ground beetles whether it fed on one or two *H. coagulata* adults using SCAR marker HcF5/HcR7, whereas SCAR marker HcF6/HcR9 was more efficient. The detection efficiency was slightly higher for ground beetles that fed on two *H. coagulata* adults (25.0%) than on one (9.10%) at 0 h digestion (Table 2).

The detection efficiency of the markers targeting the multicopy mitochondrial genes (COI and COII) was significantly higher than the single-copy SCAR markers (Table 2). This was observed even though the number of amplification cycles was higher with the SCAR markers. In general, the detection efficiency of COI (197 bp) was better than COII (295 bp) using markers HcCOI-F/R and HcCOII-F4/R4, respectively. At the 0 and 8 h time point of lacewings that fed on *H. coagulata* eggs, the COI marker detection efficiency was 91.7 and 86.4% efficient as compared to 83.3 and 47.6% for COII, respectively. Only the 8 h time point was highly significant. The detection efficiency was the same for both the COI and COII markers at the 4, 16, and 24 h retention intervals. The detection efficiency of earwigs that fed on *H. coagulata* eggs at 0 h was significantly higher for the COI marker (87.5%) and than the COII marker (25.0%). A similar, but non-significant, pattern was also observed in ground beetles that fed on adult *H. coagulata*. The detection efficiency was slightly higher with the COI marker in ground beetles that fed on one adult *H. coagulata*, 33.3% for COII as compared to 54.5% for COI. The detection efficiency reached 100.0% for the COI marker with ground beetles that fed on two *H. coagulata* adults. In both types of markers (SCAR and mitochondrial), a direct correlation between detection efficiency and amplification product size was observed.

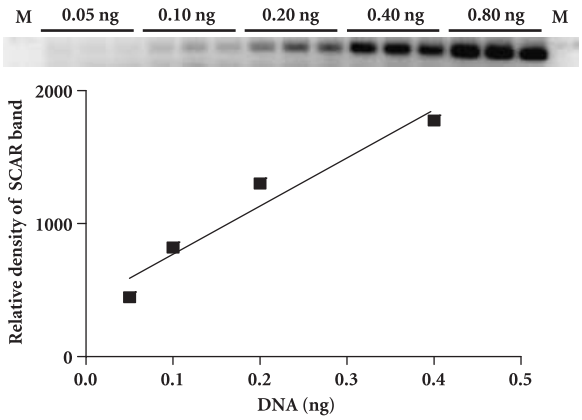

Figure 4 Representative SCAR-PCR sensitivity assay using stock genomic DNA. *Homalodisca coagulata* genomic DNA was varied from 0.05 to 0.80 ng with each point in triplicate determination (inset). *Homalodisca coagulata* DNA was utilized with HcF6/HcR9 *H. coagulata*/*H. liturata*-specific markers (166 bp). As the highest amount of genomic DNA (0.8 ng) did not fall within the linear portion of the curve (saturated), it was eliminated from the analysis.

For lacewings that fed on *H. liturata* eggs the detection efficiency was between 80 and 90% at 0 h with both *H. liturata*- and *H. coagulata*/*H. liturata*-specific markers. The *H. coagulata*/*H. liturata*-specific COII marker (G/S-COII-F/R) that produced an amplification product size of 178 bp was slightly more efficient than the COII marker (Hl-COII-F/R) that produced a size of 295 bp (90 and 80%, respectively).

Discussion

Direct field estimates of predator–prey interactions can be disruptive to the normal foraging process (Hagler et al.,

Table 2 Detection efficiency[1] (percentage, %) of molecular diagnostic markers in predators; small scale analysis. The specificity of the marker and the expected size of the DNA fragment are included below the marker name. Lacewings and earwigs fed on *Homalodisca coagulata* eggs and ground beetles fed on *H. coagulata* adults. Individual lacewings for the retention time experiment fed on 2–3 eggs, as did lacewing feeding on *Homalodisca liturata* eggs. Individual earwigs fed on 5–20 eggs. np, not performed; n/a, not applicable. Numbers in parenthesis are number of individuals tested. Shown in brackets are the sensitivity limits of the diagnostic markers measure as pg of DNA. Statistics were performed with Fisher's exact test using two-sided P values. Hc, *H. coagulata*; Hl, *H. liturata*

	HcF5/R7[2] Hc 302 bp	HcF6/R9[2] Hc/Hl 166 bp	HcCOII-F4/R4[3] Hc 295 bp	G/S-COII-F/R[3] Hc/Hl 178 bp	HcCOI-F/R Hc 197 bp	Hl-COII-F/R Hl 295 bp
Hc	[100]	[50]	[25]	[50]	[6]	[400]
Lacewing[4]						
0 h	8.3 (12)a	58.0 (12)b	83.3 (12)b	83.3 (12)b	91.7 (12)b	n/a
4 h	np	np	27.3 (11)a	18.2 (11)a	27.3 (11)a	n/a
8 h	np	np	47.6 (21)a	86.4 (22)b	86.4 (22)b	n/a
16 h	np	np	37.5 (8)a	50.0 (8)a	37.5 (8)a	n/a
24 h	np	np	9.10 (11)a	9.10 (11)a	9.10 (11)a	n/a
Lacewing[5]	25.0 (4)	50.0 (4)	50.0 (4)	50.0 (4)	50.0 (4)	n/a
Earwig[5]	12.5 (8)a	12.5 (8)	25.0 (8)a	25.0 (8)a	87.5 (8)b	n/a
Beetle[5]:						
1 adult Hc	0.0 (11)a	9.10 (11)	33.3 (12)c	16.7 (12)b	54.5 (11)c	n/a
2 adult Hc	0.0 (8)a	25.0 (8)	87.5 (8)b	100.0 (8)b	100.0 (8)b	n/a
Hl						
Lacewing[4]	n/a	80.0 (10)	n/a	90.0 (10)	n/a	80.0 (10)

[1]Values within each row followed by the same letter are not significantly different (P>0.05).
[2]Primers designed towards same SCAR sequence.
[3]Primers designed towards same COII (*H. coagulata*) sequence.
[4]Crude DNA extract procedure.
[5]QIAGEN preparation of DNA of insects at 0 h.

1992). To overcome this pitfall, indirect methods of predator assessment, such as DNA or ELISA-based techniques, can be utilized to identify prey remains in the guts of field-collected predators (reviewed in Symondson, 2002). Here we report the development of molecular diagnostic markers aimed at detecting DNA of *H. coagulata* and *H. liturata* in predator gut contents. The two types of diagnostic markers developed are single-copy SCAR markers and gene-specific markers directed towards multicopy mitochondrial genes (COI and COII). DNA amplification with the SCAR marker HcF5/HcR7 demonstrated high specificity towards *H. coagulata*, whereas the SCAR marker HcF6/HcR9 demonstrated high specificity towards the two *Homalodisca* species present in California. The SCAR markers can be used to distinguish *H. coagulata* from other closely related sharpshooter species, including *H. liturata*. In combination with the crude DNA extraction procedure, these markers can also be used to rapidly identify these sharpshooter species at the egg stage level, thus saving the time and expense required for rearing the insects to the adult stage to be properly identified. In addition, having a rapid crude DNA extraction procedure is also important because it allows for rapid screening of thousands of field-collected specimens, thus, again saving cost on labour and on the more expensive DNA extraction kits. The ability to rapidly distinguish between *H. coagulata* and *H. liturata* becomes important when monitoring the dispersal of *H. coagulata* into previously non-infested areas or counties. This will allow workers to make decisions more rapidly on how to implement the best control strategy. *Homalodisca coagulata*/*H. liturata*-specific SCAR marker HcF6/HcR9 was slightly more efficient at detecting *H. liturata* eggs in lacewings than *H. coagulata* eggs. An inverse relationship between detection efficiency and DNA fragment size was observed between the two SCAR markers, with the marker HcF6/HcR9 producing the smaller fragment having a slightly higher detection efficiency. The present results agree with recent studies that demonstrated the same pattern of detectability with regard to fragment length (Agustí et al., 1999b, 2000, 2003b; Chen et al., 2000).

The molecular markers designed towards the multicopy mitochondrial genes (COI and COII) were highly specific

towards the target pests. The mitochondrial markers had, in general, a higher detection efficiency rate than the single-copy SCAR markers. The observation that targeting multicopy genes improved prey detection efficiency was first made by Zaidi et al. (1999) with nuclear esterase genes. The first predation experiment in which both predator and prey were invertebrates was performed by Chen et al. (2000) targeting the COII genes of six species of cereal aphids. Other authors (Hoogendoorn & Heimpel, 2001; Agustí et al., 2003a, 2003b) have also shown that multicopy genes considerably increase the probability and duration of prey detection in predator guts.

The amplification reactions with the COI and COII markers were performed with 31 and 35 cycles, respectively. As explained in the methods and materials, the reaction conditions for each marker were critical in order to prevent cross-reactivity between the sharpshooter species. Due to the smaller amplification product size of the COI markers, it would be expected that these markers would have a higher detection efficiency; but this marker may also have a higher detection efficiency due to the fact that it is about 4.2-fold more sensitive than the COII marker when tested using stock genomic DNA. The sensitivities of the markers were 6.0 and 25.0 pg of DNA for the COI and COII markers, respectively. In the current data, there appears to be a correlation between sensitivity and detection efficiency. The sensitivity of the markers increases the detection of prey DNA in the predator guts. Note that this has nothing to do with competition with predator DNA, which is a separate issue (discussed in the following text). A possible explanation may be that the sensitivity of the markers is associated with accessibility of the target DNA (COI gene) within the cell, the ability of the primers to hybridize or rather the binding efficiency of the primers to target the DNA of interest, and the size of the amplification product may also lead to factors making the markers more sensitive by making *Taq* DNA polymerase extend more efficiently during the reaction. Therefore, sensitivity is distinct from detection efficiency, but contributes to the efficiency of the markers. Detection efficiency, or rate of detection, incorporates the complex environment of the predator gut contents or rather the factor of digestion comes into play. Different species digest prey proteins or DNA at different rates, and these rates are dependent on several factors (e.g., quantity consumed, temperature, time, meal size, and copy number of the target DNA) (Hagler & Naranjo, 1997; Agustí et al., 1999a; Chen et al., 2000). In short, it is important to choose the most sensitive marker because the DNA of the prey in the predator gut content is undergoing digestion, therefore decreasing the amount of detectable target DNA.

The inclusion of an internal PCR control designed for 28S is critical for PCR gut content analyses because it allows for the monitoring of the amplification reactions. We used this control to determine the presence of suitable DNA in extractions where the insects were small and to control for the presence of PCR inhibitors and failures. In other words, if a negative reaction occurs for a predator, but a positive reaction occurs for the 28S control, we can be certain that the predator was negative for the target prey or it was beyond the limits of detection and not due to the role of PCR inhibitors, failures, or lack of sharpshooter DNA in the sample. Other investigators have targeted conserved genes as an internal positive control. For example, the mitochondrial 12S rDNA (Hoogendoorn & Heimpel, 2001) and the actin gene (Zaidi et al., 1999) were used to determine the presence of pest DNA in predator guts.

In the present experimentation, the 4 h time point or postfeeding data with the molecular markers do not follow the normal expected trend of a typical decay curve, and therefore this time point could be considered off or aberrant. The exact reason for this unexpected trend is unknown at this point. Human handling errors, technical errors, or sample size could be some of the reasons to help explain these results. Further testing of these markers may be necessary at the different time points, some of which are ongoing. But the fact that these molecular markers are highly specific towards their targets (*H. coagulata* and *H. liturata*), are highly sensitive, and do not cross-react with any other sharpshooters, predators, or the additional homopterans tested in the present study increases our confidence for their intended use.

In conclusion, the development of diagnostic markers for *H. coagulata* will allow researchers to begin to understand the ecology of *H. coagulata*–predator interactions in natural environments. This information will be included in an areawide pest management approach to aid in controlling this devastating pest. Large-scale field studies to identify key predators of *H. coagulata* are in progress (V Fournier, JR Hagler, KM Daane, JH de León, RL Groves, HS Costa & TJ Hennebery, unpubl.).

Acknowledgements

We acknowledge Marissa González, Lisa A. Ledezma, and Rosa I. Ruiz from USDA, ARS (Weslaco, TX, USA), Elaine Shapland from Berkeley, CA, USA, and Juanita Corza and Chrissie Pflipsen from USDA-ARS (Phoenix, AZ, USA) for their excellent technical assistance. We thank Alexander Purcell for specimens of sharpshooters. We thank Thomas R. Unruh and Nina Bárcenas from USDA, ARS, YARL (Wapato, WA, USA) and Matthew H. Greenstone from USDA, ARS, Insect Biocontrol Laboratory (Beltsville, MD,

USA) for reading and improving a previous version of this manuscript. This work was funded by the USDA-ARS, The California Department of Food and Agriculture (CDFA), and The University of California's Pierce's disease grant program. Lastly, we thank the anonymous reviewers for improving the manuscript.

Disclaimer

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

Agustí N, Aramaburu J & Gabarra R (1999a) Immunological detection of *Helicoverpa armigera* (Lepidoptera: Noctuidae) ingested by heteropteran predators, time-related decay and effect of meal size on detection period. Annals of the Entomological Society of America 92: 56–62.

Agustí N, De Vicente MC & Gabarra R (1999b) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. Molecular Ecology 8: 1467–1474.

Agustí N, De Vicente MC & Gabarra R (2000) Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. Insect Molecular Biology 9: 263–268.

Agustí N, Shayler SP, Harwood JD, Vaughan IP, Sunderland KD & Symondson WOC (2003a) Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. Molecular Ecology 12: 3467–3475.

Agustí N, Unruh TR & Welter SC (2003b) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. Bulletin of Entomological Research 93: 179–185.

Almeida RPP & Purcell AH (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). Journal of Economic Entomology 96: 264–271.

Black IVWC, DuTeau NM, Puterka GJ, Nechols JR & Pettorini JM (1992) Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids (Homoptera: Aphididae). Bulletin of Entomological Research 82: 151–159.

Brlansky RH, Timmer LW, French WJ & McCoy RE (1983) Colonization of the sharpshooter vectors, *Oncometopia nigricans* and *Homalodisca coagulata*, by xylem-limited bacteria carriers of xylem-limited bacterial disease. Phytopathology 73: 530–535.

CDFA (2003) Pierce's Disease Program Report to the Legislature. California Department of Food and Agriculture, Sacramento, CA, USA.

Chen Y, Giles KL, Payton ME & Greenstone MH (2000) Identifying key cereal aphid predators by molecular gut analysis. Molecular Ecology 9: 1887–1898.

Greenstone MH & Morgan CE (1989) Predation on *Heliothis zea* (Lepidoptera: Noctuidae): an instar specific ELISA assay for stomach analysis. Annals of the Entomological Society of America 82: 45–49.

Hagler JR, Brower AG, Tu Z, Byrne DN, Bradley-Dunlop D & Enriquez FJ (1993) Development of a monoclonal antibody to detect predation of the sweetpotato whitefly, *Bemisia tabaci*. Entomologia Experimentalis et Applicata 68: 231–236.

Hagler JR, Cohen AC, Bradley-Dunlop D & Enriquez FJ (1992) Field evaluation of predation of *Lygus hesperus* (Hemiptera: Miridae) using a species- and stage-specific monoclonal antibody. Environmental Entomology 21: 896–900.

Hagler JR, Cohen AC, Enriquez FJ & Bradley-Dunlop D (1991) An egg-specific monoclonal antibody to *Lygus hesperus*. Biological Control 1: 75–80.

Hagler JR & Naranjo SE (1994) Qualitative survey of two Coleopteran predators of *Bemisia tabaci* (Homoptera: Aleyrodidae) and *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) using a multiple prey gut content ELISA. Biological Control 23: 193–197.

Hagler JR & Naranjo SE (1997) Measuring the sensitivity of an indirect predator gut content ELISA detectability of prey remains in relation to predator species, temperature, time, and meal size. Biological Control 9: 112–119.

Harper GL, King RA, Dodd CS, Harwood JD, Glen DM et al. (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. Molecular Ecology 14: 819–827.

Hill BL & Purcell AH (1995) Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathology 85: 209–212.

Hoogendoorn M & Heimpel GE (2001) PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. Molecular Ecology 10: 2059–2067.

Hopkins DL & Mollenhauer HH (1973) Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179: 298–300.

Hopkins DL (1989) *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. Annual Review of Phytopathology 27: 271–290.

Hoy MA (1994) Insect Molecular Genetics, an Introduction to Principals and Applications. Academic Press, San Diego, CA, USA.

Juen A & Traugott M (2005) Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. Oecologia 142: 344–352.

de León JH & Jones WA (2004) Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by polymerase chain reaction-based DNA fingerprinting methods. Annals of the Entomological Society of America 97: 574–585.

de León JH, Jones WA & Morgan DJW (2004) Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing

Pierce's disease in grapevines. Annals of the Entomological Society of America 97: 809–818.

Moran NA, Dale C, Dunbar H, Smith WA & Ochman H (2003) Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environmental Microbiology 5: 116–126.

Nielson MW (1968) The leafhopper vectors of phytopathogenic viruses (Homoptera, Cicadellidae) taxonomy, biology, and virus transmission. USDA Technical Bulletin 1382: 81–84.

Paran I & Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics 85: 985–993.

Pooler MR, Myung IS, Bentz J, Sherald J & Hartung JS (1997) Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. Letters in Applied Microbiology 25: 123–126.

Redak RA, Purcell AH, Lopes JRS, Blua MJ, Mizell RF III & Anderson PC (2004) The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relationship to disease epidemiology. Annual Review in Entomology 49: 243–270.

Sambrook J & Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schenk D & Bacher S (2004) Detection of shield remains in predators using a monoclonal antibody. Journal of Applied Entomology 128: 273–278.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H & Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651–701.

Sorensen JT & Gill RJ (1996) A range extension of *Homalodisca coagulata* (Say) (Hemiptera: Clypeorrhyncha: Cicadellidae) to southern California. Pan-Pacific Entomology 72: 160–161.

Symondson WOC (2002) Molecular identification of prey in predator diets. Molecular Ecology 11: 627–641.

Symondson WOC & Liddell JE (1996) A species-specific monoclonal antibody system for detecting the remains of field slugs, *Deroceras reticulatum* (Mueller) (Mollusca: Pulmonata), in carabid beetles. Biocontrol Science and Technology 6: 91–99.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Triapitsyn S, Mizell RF, Bossart JL & Carlton CE (1998) Egg parasitoids of *Homalodisca coagulata* (Homoptera: Cicadellidae). Florida Entomologist 81: 241–243.

Turner WF & Pollard HN (1959) Life histories and behavior of five insect vectors of phony peach disease. USDA Technical Bulletin 1188, 28pp.

Varela LG, Smit RJ & Phillips PA (2001) Pierce's Disease. University of California Agricultural Natural Research – Publication 21600.

Vega FE, Davis RE, Barbosa P, Dally EL, Purcell AH & Lee IM (1993) Detection of plant pathogen in a nonvector insect species by the polymerase chain reaction. Phytopathology 83: 621–624.

Werren JH, Windsor D & Guo L (1995) Distribution of *Wolbachia* among neotropical arthropods. Proceeding of the Royal Society of London. Series B 262: 197–204.

Yasukochi Y (1998) A dense genetic map of the silkworm, *Bombyx mori*, covering all chromosomes based on 1018 molecular markers. Genetics 150: 1513–1525.

Young DA (1958) A synopsis of the species of *Homalodisca* in the United States (Homoptera: Cicadellidae). Bulletin of the Brooklyn Entomological Society 53: 7–13.

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J & Symondson WOC (1999) Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Molecular Ecology 8: 2081–2020.